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PATENT SPECIFICATION

DRAWINGS ATTACHED

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COMPLETE SPECIFICATION

Improvements in and relating to Lipases

We, LABORATOIRE D'ANALYSES ET DE RECHERCHES BIOLOGIQUES MAUVERNAY — CENTRE EUROPEEN DE RECHERCHES MAUVERNAY (C.E.R.M.), of Riom, Puy-de-Dome, France, and SOCIETE D'ETUDES ET D'APPLICATIONS BIOCHEMIQUES, of 30 Rue de Versailles, Jouy-en-Josas, Seine-et-Oise, France, both French Bodies Corporate, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement: —

It is known that the lipases are very important enzymes in the metabolism of lipids since they cause in particular the hydrolysis of triglycerides which are transformed into diglycerides, monoglycerides, glycerol and fatty acids. A product of this type thus has a therapeutic application in the treatment of gastro-intestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies and the like.

However, the only lipases used until now have been products with a basis of ground, dried pancreas, which have numerous disadvantages. The disadvantages include a very limited activity because of the origin of the lipases, their field of activity being limited to a pH of 7.5—9.5, and, in particular, their considerable instability which, when combined with an unpleasant taste and smell, means that the product is extremely poorly suited to pharmaceutical requirements.

The new lipase according to the present invention is essentially distinguished from these former products in that to a large extent it avoids these disadvantages by reason of its different origin.

This new lipase results in fact from the culture of a micro-organism of certain species, from which it is easily isolated since it is extracellular. It is very active, very stable, and free of unpleasant smell.

It is true that certain authors have already mentioned the lipase character of certain fungus cultures, and have therefore made the hypothesis of the formation of lipases in those cultures. However, in most cases, it has been impossible to isolate the lipases by reason of their endocellular character and their remarkable properties have not been ascertained. Attempts at isolation have never gone beyond the laboratory scale, so that production on an industrial scale has never been considered.

The lipase according to the invention is thus clearly distinguished from the products formerly known or described and thus constitutes a new product which may have a useful therapeutic application in the above-mentioned field.

The characteristics of the lipases will be described in detail hereinafter, but it should be noted that it is not possible exactly to state their constitution, so that a new lipase will be identified on the one hand by the process by which it is obtained, and particularly by the nature of the micro-organism cultivated and by the culture medium, and on the other hand by its biological properties.

In the first place the invention relates to the selection of micro-organism species, the culture of which leads to the new lipase. Only certain species have in fact been shown to be capable of producing this lipase as can be seen from the following Table 1.

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riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid and inositol are favourable to the development of the fungus and to the production of the lipase, but are not indispensable. The pH of the medium before seeding should be between 5 and 7. It may be adjusted by means of ammonia or 2N sulphuric acid.

The cultures are effected as deep cultures with aeration and stirring. The two factors may vary as a function of the installation, but it should be noted that too great an aeration may harm the production of the lipase.

Throughout the duration of the culture the medium is maintained at a temperature of 20 to 35°C.

Culture is continued until the enzymatic activity reaches a certain level, i.e. until the same number of lipase units are obtained for two successive measurements effected at hourly intervals.

At the end of the culture, the liquid is filtered to eliminate the mycelium. The liquid phase is then collected for the extraction of the lipase. Since this lipase is soluble in water but insoluble in organic solvents and in concentrated aqueous salt solutions, it may easily be extracted either by precipitation by an organic solvent miscible with water (preferably acetone), or by being salted out, preferably with ammonium sulphate.

The precipitate thus obtained is centrifuged and is then dried under vacuum.

In order to increase activity and reduce the quantity of impurities in the raw product thus obtained, it is possible to make a further solution and to reprecipitate the product, for example by fractional precipitation using either solvent or salts.

After drying, a powdered product is obtained which has a very high lipase activity, the titre of which varies between 5,000 and 12,000 lipase units per gram, according to the richness of the filtrates of the culture and the number of precipitations.

It will be seen below that there are purification processes, within the scope of the invention, yielding products of even higher activity, which may reach as much as 3,000,000 units per gram.

Thirdly, the invention relates to the new lipase thus obtained which is in the form of an amorphous powder and soluble in water (the pH of the aqueous solution being approximately 5.5) and in the usual buffer solutions; insoluble in alcohol, ether, organic solvents and very concentrated solutions of mineral salts.

This lipase is essentially characterised by its biological properties which will be enumerated hereinafter with reference to annexed drawings accompanying the Provisional Specification, in which:

Figure 1 represents the stability curves of the lipase in 1 mg/ml solution, as a function of time and different temperatures; the curves 1, 2, 3, and 4 show respectively the variations in lipase activity as a percentage which is a function of the time in hours, at 50, 37, 20 and 0°C;

Figure 2 shows the action of the incubation temperature (in °C) on the activity (in %) of the lipase obtained;

Figure 3 shows the action of the powdered lipase on the different substrates, measured in micro-moles of sodium hydroxide as a function of the pH, the curves 1, 2 and 3 showing respectively the action of 50 units of lipase on three grams of olive oil, the action of 150 units of lipase on three grams of tributyrin and the action of 50 units of lipase on 3 grams of triolein;

Figure 4 shows the influence of the metal ions on the activity of the lipase, expressed in activity % as a function of the metal concentration of the enzyme-substrate mixture, curves 1 to 5 referring respectively to the action of ions of zinc, mercury, cupric copper, ferric iron and cobalt; and

Figure 5 shows the action of bile on the lipase, expressed in variations in activity (%) of five mg of powder as a function of the concentration (%) of bile salts in the enzyme-substrate mixture, curves 1, 2 and 3 referring respectively to the action of desoxycholic acid, sodium taurocholate and sodium cholate.

The stability of the lipase was first studied.

In a dry state the enzymatic powders obtained did not undergo any loss of activity after several months of storing in an oven at 37°C.

In an aqueous solution of 1 mg/ml, the stability of this lipase was studied, first as a function of the temperature for variable periods with the result as given in Table II.

TABLE IV

Temperature of Incubation (°C.)	Activity (%)
18	36
30	80
37	100
40	96
45	86
50	70

The third study related to the activity of the lipase on different substrates as a function of the incubation pH. The substrates were successively olive oil, tributyrin and triolein.

In each case an emulsion of the substrate was prepared in a solution of 3% polyvinyl alcohol, in the same manner as for the measurement on the olive oil emulsion. Tests are carried out in a similar manner in each of the three cases, viz.:

to 7.5 ml of emulsion are added 5 ml of 0.1 M calcium chloride solution, 72.5 ml of distilled water at 37°C and the mixture is brought to the pH desired. 5 mg (50 units) of enzyme in a 1 mg/ml solution which has been previously brought to the desired pH is then added. The mixture is kept at 37°C and at the required pH for 10 minutes. 10 ml of ethanol is then added, the pH is brought to 9 and the quantity of 0.1 N sodium hydroxide used is measured, including that which was used to maintain the pH during the enzymatic reaction.

A control test is first effected on a solution without enzyme kept for 10 minutes at 37°C, which is rendered inactive by 10 cc of ethanol, to which is added the enzymatic solution (after the ethanol). The results obtained respectively with the three previously mentioned substrates are shown in Tables V, VI, and VII as well as in Figure 3 of the drawing.

TABLE V

pH	Number of Micro-Moles of Sodium Hydroxide used for the Regulation	Total Number of Micro-Moles of Sodium Hydroxide used
2.25	0	0
3.30	0	149
4.05	0	101
4.95	0	79
7.00	105	209
7.50	162	183
9.00	23	23

Finally the action of the lipases as a function of various inhibitors, activators and protectors was conducted with the following results:

a) Mineral compounds

Tests on ions of zinc, mercury, copper, lead, tin, iron, cobalt, aluminium, strontium, magnesium and manganese were carried out in the presence of calcium according to the usual method of titrating metals. In the case of calcium, which is a strong activator, measurement was carried out by the usual method but without calcium, and an arbitrary value of 100% was given to the activity obtained, the concentration of 5.5×10^{-3}M, which gives an activity of 466% with respect to the measurement without calcium, corresponds exactly to the quantity of calcium normally used in the measuring process.

The results obtained are shown in Figure 4.

Calcium, besides its high activating power, also has a protective power for the aqueous lipase solutions. Thus a 1 mg/ml lipase solution in a 0.1 M solution of calcium chloride does not undergo any loss of activity after 6 hours at 18°C, while in the absence of calcium the same solution loses 9 to 12% of its activity.

b) Organic compounds.

Different tests were conducted using 'Complexon III" (registered Trade Mark), human albumin, potassium ferrocyanide, cholesterol, urea, Tween 20, sodium laurylsulphate, glycerol, bile salts, and oleic acid

The following results were obtained:

(i) Action of Complexon III (ethylene diamine tetra-acetic acid): 100% activity was given for the measurements in the absence of Complexon, said activity is maintained in the presence of a concentration of 1.1×10^{-4}N, and is lowered to 85% for a concentration of 5.5×10^{-4}N, to 78% for a concentration of 1.1×10^{-3}N and to 15% for a concentration of 5.5×10^{-3}N for which all the calcium is complexed.

(ii) Action of human albumin, regarding 100% activity as the measurement in the absence of albumin, it is found that in the presence of 1 ml of a 20% aqueous albumin solution this activity is increased to 141% and in the presence of 5 ml of this solution it is brought to 158%.

(iii) Potassium ferrocyanide does not effect either activation or inhibition.

(iv) Cholesterol is a slight lipase activator.

(v) Urea does not effect any change on the enzymatic activity.

(vi) Tween 20 and sodium laurylsulphate both have one characteristic feature: in small quantities they are activators but in greater quantities they become inhibitors as shown by Tables VIII and IX.

TABLE VIII

Concentration of Tween 20 in the Enzyme-Substrate Mixture (γ per ml.)	Activity (%)
0	100
50	105
100	124
150	148
200	100
300	0

TABLE IX

Concentration of Laurylsulphate in the Enzyme-Substrate Mixture (γ per ml)	Activity (%)
0	100
100	111
1000	29

may either be employed alone or in association with gastric extracts, bile extracts and hemicellulase.

Finally, it is important to note that still more purified forms of this lipase can be obtained, and can be employed in other applications. These forms are the following:—

Purified forms assaying at 50,000 to 150,000 units per gram are obtained from forms assaying at 5,000 to 15,000 units per gram by fractional precipitation, by the elimination of inactive residues (an operation which can be combined with dialyses); the precipitation being carried out at low temperature (of the order of $-5°$ to $+5°C$) and the most convenient solvent, but not exclusively, being acetone, other solvents being lower aliphatic alcohols.

Preparations assaying at 5,000, 12,000, 50,000 to 150,000 units per gram can be used in therapeutics in the following forms:

— simple tablets (excipient: cornflour, talc)
— enteric tablets (glutenised, isokeratol)
— effervescent tablets (excipient: bicarcarbonate of soda, citric acid or other excipient which, dissolved in water, releases CO_2)
— capsules (excipient: paraffin oil),

these different forms, containing lipase in doses of 2,500, 5,000, 25,000 or 50,000 units, and their therapeutic indications being:

— all pancreatic insufficiencies whether or or not accompanied by steatorrheas,
— dyspepsias,
— disorder of the assimilation of lipids,
— intolerances to fats,
— dyslipemias and disorders of the absorption of lipids in gastrectomised individuals.

The usual dosage regimen is from 2 to 5 tablets or capsules per day.

Extremely purified forms assaying 2,500,000 to 3,000,000 units per gram are prepared from lipases assaying 10,000 to 30,000 units per gram which are applied to cation exchange resins, for example of the type IRC50 or Xe64, and which may be eluted by a suitable buffer, at pH between 5 and 6, the mineral element of the buffer used being then eliminated by all convenient methods, in particular by passage over a molecular sieve, for example a product of the type SEPHADEX G 25 (registered Trade Mark), or by dialysis.

These purified preparations are used in injectable form, the lipase being offered in the form of lyophilised powder, which dissolves at the time of use in a physiological saline or a buffer.

The therapeutic indications are then the following:

— xanthomatoses,
— arterio-scleroses,
— severe pancreatic insufficiencies,
— essential hyperlipidemias,
— hereditary dyslipidemias.

WHAT WE CLAIM IS:—

1. A process for the preparation of a stable lipase of high activity, characterised in that a culture is made with aeration and stirring with a species of micro-organisms chosen from Mucor Hiemalis, Rhizopus Delemar and Rhizopus arrhizus, on a nutrient medium containing organic substances, nitrogen sources and mineral elements, and if desired, vitamins, that this culture is continued until the moment when the enzymatic activity of the medium remains constant, that the culture medium is separated by filtration from the filtrate and that the lipase contained in this filtrate is collected.

2. A process as claimed in claim 1, characterised in that the lipase contained in the filtrate is collected by precipitation by means of acetone.

3. A process according to claim 1, characterised in that the lipase contained in the filtrate is collected by salting out by means of ammonium sulphate.

4. A process according to claim 2 or 3, characterised in that the lipase is prepared in solid form by centrifugation and drying under vacuum.

5. A process according to claim 4, characterised in that the lipase is purified by fractional precipitation, by a solvent chosen from acetone and lower aliphatic alcohols at a low temperature between $-5°C$ and $+5°C$.

6. A process according to claim 4 or 5, characterised in that the lipase is purified by passage of its solution over a cation exchange resin, by dialysis or by passage over a molecular sieve.

7. A new lipase for therapeutic application, characterised in that it results from the application of a process according to any of claims 1 to 6.

8. A lipase according to claim 7, suitable for the treatment of digestive disorders due to pancreatic insufficiency, secretory insufficiencies of senescence, of atheromatosis, psoriasis, hyperlipidemias and dyslipidemias, assaying at 5000 to 150,000 units per gram.

9. A dosage unit form of a lipase according to claim 8, each unit containing from 2500 to 50,000 units of said lipase.

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1084431 PROVISIONAL SPECIFICATION
2 SHEETS *This drawing is a reproduction of the Original on a reduced scale*
Sheets 1 & 2

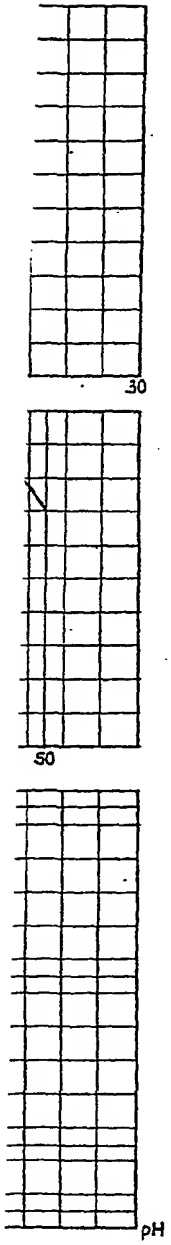

Fig.4

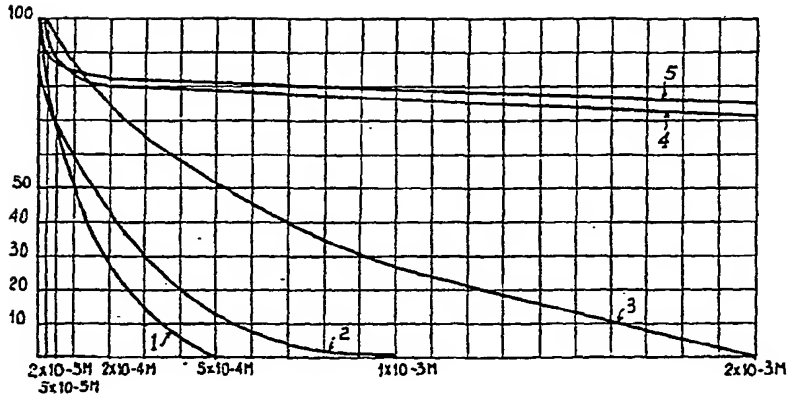

Fig.5